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# Immunofluorescent localisation of enterokinase in human small intestine

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SUMMARY The distribution of enterokinase in human intestine was studied in operative mucosal biopsies using specific antiserum to human enterokinase, previously purified to apparent homogeneity by affinity chromatography and immunoabsorption. Fluorescence was observed in the brush-border and glycocalyx of the duodenum and proximal 15 cm of jejunum distal to the D/J flexure. Distal jejunum and ileum as well as stomach and colon were consistently negative. Brunner's glands and goblet cells were never stained by specific antibody. Preliminary evidence was obtained that the human enterokinase molecule contains a specific antigenic determinant in its polypeptide component and a second determinant in the oligosaccharide moiety which cross-reacts with blood group A. Preliminary evidence was also obtained that mucosal synthesis of enterokinase may be impaired in jaundice due to carcinoma of the pancreas and induced in the small intestine distal to the normal limit of synthesis after pancreatico-duodenectomy.

Enterokinase is the glycoprotein enzyme in the small intestine that triggers the activation of the zymogens in pancreatic juice by converting trypsinogen to trypsin; trypsin then activates the other zymogens on which enterokinase has no effect. In the absence of enterokinase, the zymogens remain unactivated and protein deficiency results (Hadorn *et al.*, 1969; Tarlow, 1970). Enterokinase has therefore a key permissive role in protein digestion and plays an essential part in the zymogen mechanism that prevents the pancreas destroying itself. Despite its apparent importance, the origin and distribution of the enzyme in human intestine has not been precisely defined (Lebenthal *et al.*, 1976).

Studies of the distribution of enterokinase catalytic activity in fractions of small intestinal mucosal homogenates from animals have suggested that the enzyme is produced by the intestinal epithelial cell and is located in the microvillus membrane (Lobley and Holmes, 1970; Hadorn *et al.*, 1971; Nordstrom and Dahlqvist, 1971; Nordstrom, 1971; Louvard *et al.*, 1973; Schneider *et al.*, 1975). These findings are supported by similar, though necessarily more limited observations on human tissue (Nordstrom and Dahlqvist, 1972; Lobley *et al.*, 1973; Schmitz *et al.*, 1974). Other workers have proposed that enterokinase may be produced by Brunner's glands or goblet cells and subsequently be bound in the glycocalyx and brush border of the enterocytes (Eggermont *et al.*, 1971; Rutgeerts *et al.*, 1972; Woodley and Keane, 1972). Lojda and Malis (1972) used a histochemical technique to demonstrate enterokinase activity in sections of intestinal epithelium including two human duodenal biopsies. The number of biopsies and the resolution of the technique was, however, insufficient to resolve these uncertainties and the only immunofluorescent study had been performed in pigs (Takano *et al.*, 1971), using antisera prepared against porcine enterokinase of low specific activity and heavily contaminated with other antigens.

Human enterokinase has recently been purified to apparent homogeneity (Grant and Hermon-Taylor, 1976) and the availability of specific antibody has enabled the distribution of the enzyme to be studied in man using a standard immunofluorescent technique.

## Methods

The purification of human enterokinase and the enzyme assays have been described in detail (Grant and Hermon-Taylor, 1975, 1976). The principal preparative step was affinity chromatography using

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glycylglycine p-aminobenzamidine substituted Sepharose-4B after which only 1% of the α-glucosidases remained. Purification was completed by immunoadsorption using antisera raised to enterokinase-free material containing these enzymes prepared as a by-product of the purification procedure. The final preparation was free of any other enzymic activity tested including aminopeptidase, maltase, sucrase, isomaltase, lactase, glucoamylase, cellobiase, trehalase, and alkaline phosphatase and appeared as a single band on polyacrylamide gel electrophoresis.

### PREPARATION OF ANTISERUM

Four hundred micrograms of highly purified human enterokinase (specific activity, the production of 4260 nmol trypsin. $min^{-1}$. $mg^{-1}$) was emulsified in Freund's complete adjuvant. The material was divided equally between two California rabbits and each injected subcutaneously at four sites. Intravenous injections of 40 μg enterokinase were given at six and eight weeks and serum obtained 10 days later. The catalytic activity of 1 μg enterokinase was neutralised by 11·5 μl of this anti-serum. Cross-reacting antibodies with blood group A and to a lesser extent B reactivity present in native enterokinase antiserum were removed by adsorption with human erythrocytes or Sepharose immobilised colonic mucus. The enterokinase neutralising ability of the antiserum was unchanged.

### TEST FOR ANTISERUM SPECIFICITY

Intestinal aminopeptidase and the α-glucosidases are very similar to enterokinase in their physicochemical properties and could not be separated from enterokinase by a combination of gel filtration, ion exchange chromatography, and preparative isoelectric focusing. It is unlikely that they were represented in the final enterokinase preparation in an enzymically inactive form while preserving their immunological identities, as their removal after affinity chromatography was itself completed by immunological means. Nevertheless, it was a possibility that needed to be explored. This could not be carried out by immunoelectrophoresis as the antienterokinase antiserum was non-precipitating when run against the highly purified enzyme. Furthermore, antibodies to intestinal aminopeptidase and the α-glucosidases did not inhibit their catalytic activities (Grant and Hermon-Taylor, 1976). The ability of immobilised antienterokinase antiserum to bind intestinal aminopeptidase and the α-glucosidases was therefore tested.

Immunoglobulin in 10 ml antienterokinase antiserum was precipitated by adding solid ammonium sulphate to 40% (w/v) saturation. The precipitate was resolubilised and dialysed against 0·1 mol sodium bicarbonate containing 0·5 M NaCl. The separated immunoglobulin was coupled to 20 g Sepharose-4B activated as described by Gospodarowicz (1972). Two columns, each of bed volume 1 ml, of the substituted Sepharose were packed and equilibrated in 50 mM Tris/HCl pH 8·5 containing 0·2 M NaCl. 7·5 ml of a solution of partially purified human enterokinase containing enterokinase (60 nmol trypsin.$min^{-1}$), aminopeptidase (150 nmol nitroaniline.$min^{-1}$), maltase (0·28 mol disaccharide. $min^{-1}$), and glucoamylase (0·1 mol disaccharide. $min^{-1}$) was washed through each column. The eluates were retained and assayed for enzymic activities. Protein was estimated by the method of Lowry *et al.* (1951). Each bed of immobilised antiserum removed 97% of the enterokinase activity in the applied sample; the specific activities of aminopeptidase and the α-glucosidases in the eluates were unchanged.

### PREPARATION AND STAINING OF TISSUES

Forty-four biopsies were obtained from 27 patients during the course of an abdominal operation. Six patients had gastric neoplasms; 13 peptic ulcer; two common bile duct stones; two carcinoma of the pancreas; one small intestinal stricture; three colorectal carcinoma. Thirty-four biopsies were from the duodenum or jejunum but the stomach, ileum, and colon were also examined. Tissues were snap frozen in isopentane-liquid nitrogen and 5 μ cryostat sections were stained by the indirect immunofluorescent technique. Anti-enterokinase antiserum (diluted 1/5 or 1/10) was applied to the sections; after 30 minutes at room temperature the sections were washed in phosphate buffered saline and fluorescein isothiocyanate sheep anti-rabbit immunoglobulin conjugate (Wellcome Reagents Ltd.) was applied for 30 minutes. Sections were mounted in glycerol and examined by ultra-violet epi-illumination. Normal rabbit serum and conjugate controls were also run.

## Results

Anti-enterokinase antiserum after absorption with blood group A and B human erythrocytes or immobilised colonic mucus showed sharply localised staining of brush border microvilli and glycocalyx of the intestinal epithelium in the duodenum and proximal jejunum (Figure; Table). Fluorescence was maximal in the cells in the apical parts of the villi. Fourteen out of 16 duodenal biopsies were positive (Table). The two negative biopsies were taken within 3 cm of the pylorus; one patient had a lymphosarcoma and the other an advanced carcinoma of the stomach. One other patient with a duodenal ulcer had weak staining in a biopsy from the first part of

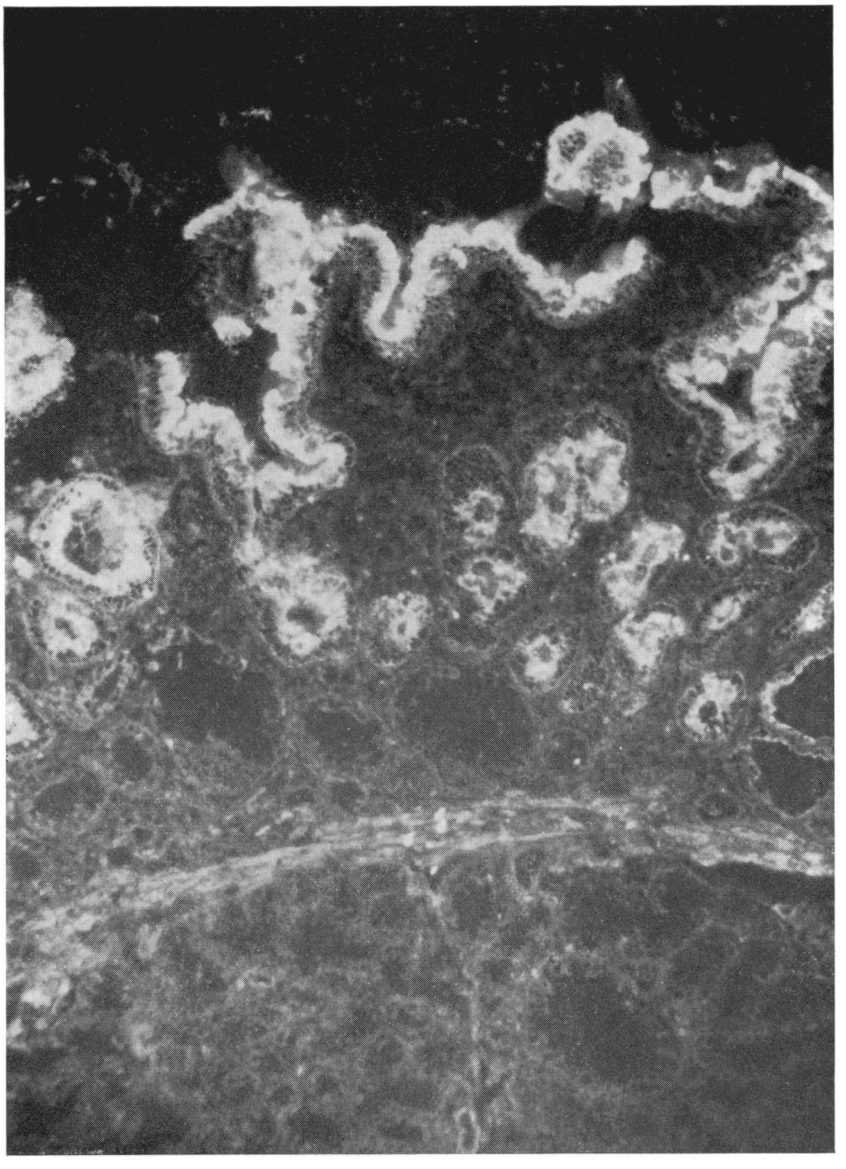

Figure a. *Photomicrograph × 10 of duodenal mucosal biopsy 3 cm distal to the pylorus from a patient with a duodenal ulcer stained for enterokinase as described in the text. Brunner's glands deep to the muscularis mucosae were consistently negative.* (*Figure continued overleaf*.)

the duodenum. Brunner's glands and goblet cells were not stained. Fluorescence was noticeably attenuated in biopsies from the second part of the duodenum in patients with obstructive jaundice due to carcinoma of the head of the pancreas. In jejunal biopsies taken within 10 cm of the duodenojejunal flexure (DJF), fluorescence was strongly positive, and, as in the duodenum, was maximal in the apical parts of the villi. Between 10 cm and 20 cm distal to the DJF, as accurately as could be measured at operation, only five out of 11 biopsies were positive; the fluorescence was weaker and sometimes more patchily distributed, the brush border and glycocalyx of some villi staining, while others were unstained.

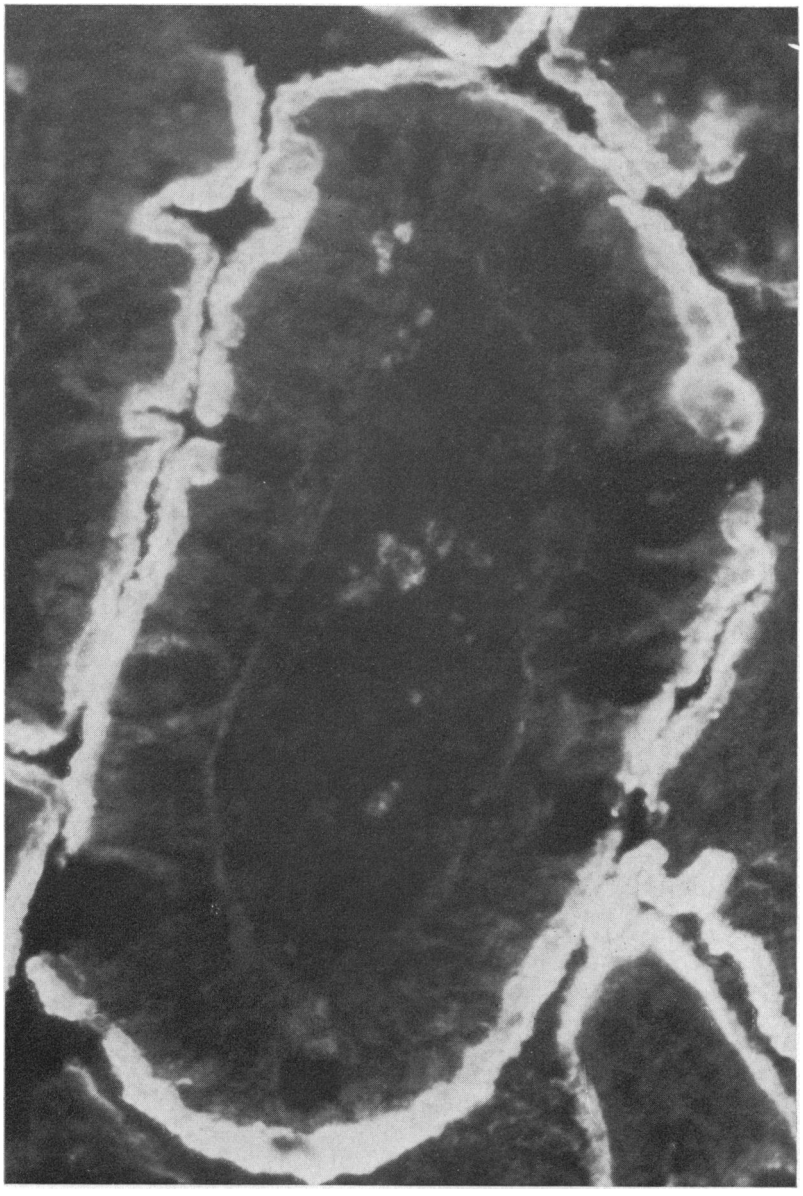

Figure b. *Photomicrograph × 40 of jejunal mucosa 8 cm distal to the duodenojejunal flexure. Enterokinase fluorescing in the brush-border and glycocalyx of the enterocytes. Staining stops abruptly at the mouth of mucus goblet cells which were consistently negative.*

Sections from more distal sites in the small intestine and from stomach and colon were consistently negative.

Rabbit anti-human enterokinase antiserum unabsorbed with group A and B erythrocytes gave the staining pattern and localisation described above, in intestinal biopsies from patients of blood group O. In blood group A patients there was additional staining of goblet cells in biopsies from any site throughout the gastrointestinal tract, including stomach, small intestine, and colon. Such goblet cell staining was completely abolished by prior absorption of the antiserum with group A erythrocytes. The staining of brush border and glycocalyx in duodenum

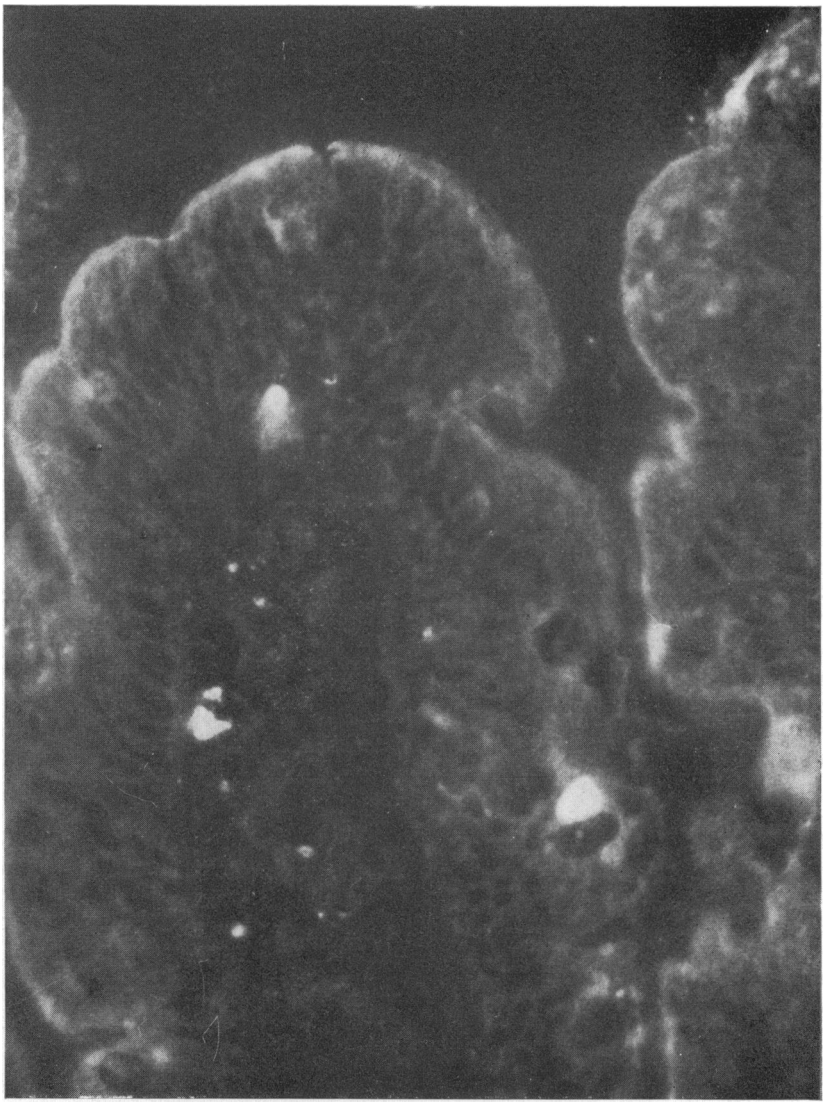

Figure c. *Photomicrograph × 40 of jejunal mucosa 20 cm distal to the duodenojejunal flexure. Negative for enterokinase staining.*

and proximal jejunum was, however, left intact, and so was the ability of the antiserum to neutralise enterokinase activity.

## Discussion

The reliance that can be placed on immunofluorescent localisation in tissues depends on the specificity of the antiserum, which is in turn related to the homogeneity of the immunising antigen. It is not possible, particularly with macromolecules, to demonstrate homogeneity, only the apparent lack of heterogeneity, but the appearance of highly purified human enterokinase as a single band on polyacrylamide gel electrophoresis stained for protein or carbohydrate, its high ceiling specific activity, the absence of any other enzymic activity tested (Grant and Hermon-Taylor, 1976), and the failure of the antiserum to bind the other, most closely related enzymes, support the reliability of the present observations.

These considerations and the finding that enterokinase contained no detectable N-acetyl-galactosamine or glucose (Grant and Hermon-Taylor, 1976)

Table *Distribution of immunofluorescent staining of brush-border and glycocalyx in biopsies of human intestinal mucosa by specific anti-human enterokinase antiserum*

| *Biopsy site* | *Brush-border fluorescence* | |
|---|---|---|
| | *Biopsies positive* | *Biopsies negative* |
| Stomach | 0 | 4 |
| Duodenum | | |
| First part | 8 | 2 |
| Second part | 4 | 0 |
| DJ flexure | 2 | 0 |
| Jejunum cm distal to DJF | | |
| 5 | 1 | 0 |
| 8 | 1 | 0 |
| 10 | 2 | 1 |
| 15 | 3 | 3 |
| 20 | 0 | 2 |
| 20-70 | 0 | 5 |
| Ileum | 0 | 3 |
| Colon | 0 | 3 |

makes it very unlikely that the highly purified enzyme was contaminated with blood group substance A or glycolipid with group A reactivity. Native anti-human enterokinase antiserum, however, stained goblet cells in the intestinal mucosa of group A subjects (and to a lesser extent group B) but not of group O; the distribution of positive goblet cell staining was similar to that described by Glynn and Holborow (1959) for intestinal blood group substances. This interesting anomaly forms the subject of a separate investigation but is at present open to the interpretation that the enterokinase molecule contains two antigenic determinants one of which is specific, responsible for immuno-inhibition, and is in the polypeptide core. The erythrocyte absorbed anti-enterokinase antiserum used to localise the enzyme in the present study would contain specific immunoglobulin directed against this determinant. The other determinant is likely to be situated in the oligosaccharide chains which, despite the absence of N-acetyl galactosamine, are able to adopt the group A configuration. A similar phenomenon is described for carcinoembryonic antigen (Fuks *et al.*, 1974) and for the $\alpha$-chain of human chorionic gonadotrophin (März *et al.*, 1973). The immunoglobulin population in native anti-enterokinase antiserum recognising this determinant and staining group A substance in gastric and intestinal goblet cells is, of course, removed by specific erythrocyte absorption. Native antiserum prepared against highly purified porcine enterokinase (Grant and Hermon-Taylor, 1975) stained goblet cells in porcine gastric and intestinal mucosa in a similar manner (unpublished observations) as well as the glycocalyx and brush border of pig duodenum and proximal jejunum. As in the human study, the cross-reacting immunoglobulin population could be absorbed from the antiserum leaving the neutralising ability for porcine enterokinase unimpaired.

Enterokinase appears to be quite localised in human intestine, a finding in agreement with the chemical observations of Eggermont *et al.* (1971) on biopsies from six normal adults. Its presence in the brush-border and glycocalyx of some of the biopsies in the present study may have been affected by the condition necessitating operation, particularly in the first part of the duodenum in patients with an advanced gastric neoplasm or an active duodenal ulcer. Fluorescence was also noticeably attenuated in the second part of the duodenum in the presence of jaundice due to carcinoma of the head of the pancreas. In no case, however, was fluorescence observed in biopsies taken more than 15 cm distal to the duodeno-jejunal flexure.

Enterokinase is clearly not produced in goblet cells or Brunner's glands in an immunologically recognisable form; the present investigation strongly supports the interpretation of previous histochemical and chemical observations (Lojda and Malis, 1972; Nordstrom and Dahlqvist, 1972) that the enzyme is synthesised in the intestinal epithelial cell. As the enterocyte migrates from the crypt to the apex of its villus and matures, enterokinase accumulates, accounting for the differential villous distribution of the enzyme; this is similar to the concentration of intestinal digestive enzymes towards the apical part of the villi also reported in man (Nordstrom and Dahlqvist, 1973).

Since all the enterocytes lining the intestine must presumably contain the gene coding for enterokinase, it is interesting to speculate on the nature of the signal responsible for confining genetic expression to the duodenum and proximal few centimetres of jejunum. Synthesis might, for example, be signalled by a critical luminal concentration of trypsin and chymotrypsin or bile acids, since these have been shown to liberate enterokinase from intestinal microvilli (Hadorn *et al.*, 1971; Nordstrom, 1972) and reduced trypsinogen activation has been identified in children with biliary atresia (Hadorn *et al.*, 1974). If this were so, induction of enterokinase could be expected to occur if jejunum beyond the normal limit of enterokinase synthesis were anastomosed to the bile and pancreatic ducts. We have recently identified enterokinase in endoscopic biopsies of jejunal mucosa from a patient 13 years after a Whipple's operation, in whom the jejunum had been divided 20 cm beyond the flexure and anastomosed proximally in such a way.

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